# IMPACT OF TRANSPLANTATION OF HIF-1A TRANSFECTED NEURAL STEM CELLS ON NF200 AND GFAP EXPRESSION IN INJURED SPINAL CORD OF RATS

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***ABSTRACT***

*This study aimed to investigate impact of transplantation of hypoxia inducible factor-1α (HIF-1α) transfected neural stem cells (NSCs) on expressions of neurofilament 200 (NF200) and glial fibrillary acidic protein (GFAP) in injured spinal cord of rats. Spinal cord injury (SCI) was induced in rats by using an electrically controlled device. A total of 120 SD rats were randomly divided into 4 groups: sham group, SCI group, NSC group and HIF-NSC group (n=30 per group). The expressions of HIF-1α, NF200 and GFAP in injured spinal cord were detected by immunohistochemical staining. Optical density (OD) of HIF-1α positive cells in HIF-NSC group was significantly higher than that in sham, SCI and NSC groups at different time points (P<0.01), and its expression peak was postponed to 14 days after transplantation. The OD of NF200 positive cells in HIF-NSC group was significantly higher than that in SCI group and NSC group 3, 7, 14 and 28 days after transplantation (P<0.05). The number of NF200 positive axons in HIF-NSC group was significantly larger than that in SCI group and NSC group 28 days after transplantation (P<0.01). At 7, 14, and 28 days after transplantation, the area of GFAP positive cells in HIF-NSC group was significantly smaller than that in SCI group and NSC group (P<0.01). Thus, the transplantation of HIF-1α transfected NSCs can significantly increase HIF-1α expression, up-regulate NF200 expression and inhibit GFAP expression in the injured spinal cord of rats. This suggests that the transplantation of HIF-1α trasfected NSCs may inhibit the proliferation of glial cells and the formation of glial scar and promote the regeneration of injured neural axons.*

***Key words**: Spinal cord injury, Neural stem cells, Hypoxia inducible factor-1α, Neurofilament 200, Glial fibrillary acidic protein.*

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## Introduction

Hypoxia inducible factor-1α (HIF-1α) is crucial for maintenance of intracellular oxygen homeostasis and regulation of gene expressions as a response to hypoxia[1, 2]. HIF-1α, which is a nuclear protein having transcriptional activity, can increase the tolerance of cells to hypoxia and it is closely related to the angiogenesis. Neural stem cells (NSCs) are easy to manipulate, and can be transfected with multiple exogenous genes achieving high expression. Thus, NSCs have been used as favorable vectors for gene therapy[3]. In our previous study, the HIF-1α cDNA was successfully cloned from human glioma[4] and used to construct recombinant adenovirus vector with adenovirus[5]. In this study, adenovirus mediated transfection of HIF-1α was performed in NSCs in vitro, which were then transplanted into rats with spinal cord injury (SCI). The protein expressions of neurofilament 200 (NF200) and glial fibrillary protein (GFAP) in the injured spinal cord were detected, aiming to explore the mechanisms underlying the NSC induced repair of SCI.

## Materials and methods

### *Recombinant plasmid and main reagents*

HIF-1α adenovirus vector was constructed in our lab. HIF-1α monoclonal antibody (Wuhan Boster Biotech Co., Ltd), NF200 monoclonal antibody (Beijing Zhongshan Biotech Co., Ltd), GFAP polyclonal antibody (Zymed, USA) and two step kit for immunohistochemistry (Zymed, USA) were used in the present study. Other reagents were domestic and analytically or chemically pure.

***Animals and grouping***

A total of 120 SD rats weighing 250±20 g were purchased from Experimental Animal Center of Wenzhou Medical Collage and randomly assigned into sham group, SCI group, NSC group and HIF-NSC group (n=30 per group). In sham group, rats received lamina incision. In SCI group, SCI was induced, and then rats received injection of normal saline (5 μl) in the spinal cord. In NSC group, SCI rats received injection of NSC suspension ($2\times10^5$/μl; 5 μl). In HIF-NSC group, SCI rats received injection of HIF-1α transfected NSC suspension ($2\times10^5$/μl; 5 μl). At 1, 3, 7, 14 and 28 days after transplantation, rats were sacrificed (n=6 per time point).

***Modeling of SCI in rats***

An electrically control device was used to cause SCI in rats[6]. Rats were intraperitoneally anesthetized with 10% chloral hydrate (0.35 ml/100 g) and then fixed in a stereotaxic instrument. A midline incision (3 cm) was made at the back with thirteenth rib as a marker. The paraspinous muscles were separated and the T13-L1 laminas were removed. The canalis spinalis was opened to the vertebral pedicle, and about 10 mm spinal cord was exposed. The T12 and L2 laminas were fixed with two clamps, and the spinal cord corresponding to T13 spinous process was injured. The posterior middle vessel severed as the center for attack. An electromagnetic programmed pontil was used to attack the spinal cord at a power of 10×2.5 g·cm (weight: 10 g; height: 2.5 cm; diameter of lower part of pontil: 2.5 mm; contact time: 0.1 s). The wound was closed. The presence of following symptoms was used to confirm the successful introduction of SCI: spinal cord edema and hemorrhage; the tail presented with spastic swing; bilateral hindlimbs showed retraction-like flutter; bilateral hindlimbs showed flaccid paralysis. After attack, penicillin was intramuscularly injected twice daily (25000 IU) for 3 days. In addition, the blabber was pressed thrice daily aiming to establish emptying reflex or urination by puncture. Three days after SCI, rats with following manifestations were included for further experiment:

1) Spastic paralysis of bilateral hindlimbs;

2) body temperature ranged from 36°C to 37°C. When rats died, additional rats were included.

***Preparation of HIF-1α transfected NSCs***

Separation of NSCs, preparation of HIF-1α transfected NSCs, identification of NSCs, transfection of HIF-1α into NSCs and detection of HIF-1α were done[7]. At 2 days before transplantation, NSCs in logarithmic phase were harvested by centrifugation and then resuspended in serum free medium to prepare single cell suspension. The cell density was adjusted to $1\times10^6$/ml. HIF-1α adenovirus vector was added to the flask containing NSCs at a MOI of 200 PFU/cells. Incubation was done for 48 h followed by centrifugation. The supernatant was removed, and single cell suspension was prepared with serum free medium. The cell density was adjusted to $2\times10^5$/μl. This cell suspension was placed on ice and used for transplantation. At the same time, NSCs at a density of $2\times10^5$/μl were prepared and placed on ice for transplantation. The residual NSCs after transplantation were subjected to trypan blue staining. Results showed the viability of NSCs was as high as 90-95%. Injection of NSCs was done with a microinjector according to previously described[8].

***Immunohistochemistry for HIF-1α, NF200 and GFAP***

At different time points, rats were sacrificed and transcardially perfused with paraformaldehyde for 30 min. The injured spinal cord (1.5 cm in length) was harvested and fixed in 4% paraformaldehyde in PBS at 4°C for 12 h. Then, the spinal cord was dehydrated in 15% sucrose in PBS and then in 30% sucrose in PBS until the tissues sunk. Sections were obtained (30 μm in thickness). Two step kit for immunohistochemistry was employed to detect the protein expressions of HIF-1α, NF200 and GFAP.

***Detection of optical density and area of positive cells***

After immunohistochemistry, sections were observed under a light microscope and analyzed with image analysis system. Ten randomly selected sections were used for the detection of optical density (OD) of positive cells in the spinal cord. Three fields were randomly selected and 5 positive cells were detected in each field. The OD of sample sections was subtracted by OD of negative control sections, and the average was obtained.

The opposite average was calculated and used as the final OD of positive cells. After immunohistochemistry for GFAP, sections were observed at a magnification of 400 and 5 fields were randomly selected to detect the area of GFAP positive cells

($\mu m^2$) followed by calculation of average.

***Statistical analysis***

Statistical analysis was performed with SPSS version 11.0. Data were expressed as mean ± standard deviation. Comparisons were done with t test between two groups or with one way analysis of variance (ANOVA) among groups. A value of $P<0.05$ was considered statistically significant.

## Results

***HIF-1α expression***

In sham group, HIF-1α expression was undetectable. In remaining 3 groups, HIF-1α positive cells were observed (Figure 1), and the OD was determined in each group (Table 1).

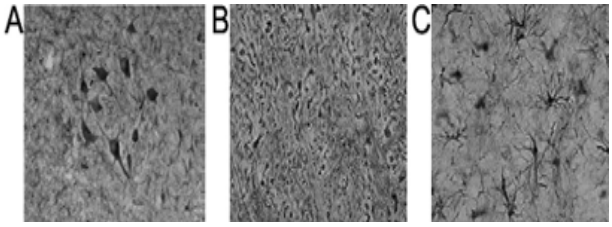


**Figure 1:** Immunohistochemistry for HIF-1α (14 d; A), NF200 (28 d; B) and GFAP (28 d; C) in HIF-NSC group (×100)

| Group | OD of HIF-1α positive cells at different time points after transplantation | | | | |
|---|---|---|---|---|---|
| | 1d | 3d | 7d | 14d | 28d |
| SCI | 40.1±3.2 | 65.6±3.7# | 53.8±3.9 | 52.2±3.0 | 37.2±3.5 |
| NSC | 42.5±4.3○ | 68.9±4.2○ | 62.1±5.1○ | 57.7±3.8○ | 32.8±2.9○ |
| HIF-NSC | 58.6±3.5*▲ | 85.6±4.4*▲ | 90.8±5.7*▲ | 105.4±5.8*▲ | 90.1±4.2*▲ |

**Table 1:** OD of HIF-1α positive cells at different time points after transplantation (x±s).

*Note: # P<0.0 vs day 1 in SCI group; ○P<0.05, * P<0.01 vs SCI group; ▲ P<0.01 vs NSC group.*

***NF200 expression***

NF200 expression was only found in the axons of neurons (Figure 1B). At different time points after transplantation, the OD value of NF200 positive axons was determined (Table 2).

***GFAP expression***

SCI can cause increase in the area of GFAP positive cells. After transplantation of NSCs or HIF-NSCs, the area of GFAP positive cells reduced significantly (Figure 1C). At different time points after transplantation, the area of GFAP positive cells is shown in Table 3.

| Group | OD of NF200 positive axons at different time points | | | | |
|---|---|---|---|---|---|
| | 1d | 3d | 7d | 14d | 28d |
| Sham | 115.8±8.2 | 116.7±7.3 | 116.4±5.9 | 115.7±4.8 | 117.4±6.5 |
| SCI | 403.±6.6 | 45.4±5.8 | 47.7±5.5 | 50.4±5.7 | 50.6±5.6 |
| NSC | 42.4±5.2 | 50.1±5.6 | 58.3±5.5△ | 65.4±6.1△ | 78.7±7.2△ |
| HIF-NSC | 44.6±6.6# | 59.7±5.4○▲# | 85.2±5.4○▲# | 90.4±6.1○▲# | 95.6±6.6○▲# |

**Table 2:** OD of NF200 positive axons in different groups at different time points (x±s).

*Note: △ P<0.05, ○ P<0.0 vs SCI group; ▲ P<0.01 vs NSC group; # P<0.01 vs sham group.*

| Group | Area of GFAP positive cells at different time points | | | | |
|---|---|---|---|---|---|
| | 1d | 3d | 7d | 14d | 28d |
| Sham | 993±65 | 1001±78 | 997±69 | 984±87 | 1004±83 |
| SCI | 678±65 | 1042±76 | 1678±99 | 1900±101 | 1794±103 |
| NSC | 654±68 | 991±79 | 1487±98# | 1408±104# | 1394±97# |
| HIF-NSC | 659±67 | 985±78 | 1268±87*○ | 1214±97*○ | 1054±93*○ |

**Table 3:** Area of GFAP positive cells in different groups at different time points (μm2, x±s).

*Note: * P<0.001 , # P<0.01 vs SCI group; ○ P<0.01 vs NSC group.*

## Discussion

HIF-1 was first extracted from the Hep3B cells undergoing hypoxic treatment by Semenza et al in 1992[9]. Studies have confirmed that HIF-1 is a nuclear transcriptional factor and a heterodimer composed of α and β subunits. The α subunit determines the biological activity of HIF-1 and the HIF-1 activity increases under the hypoxic condition. To date, more than 50 target genes of HIF-1 have been identified[10]. HIF-1 can induce the expression of glycolysis related enzymes to promote anaerobic metabolism. HIF-1 may up-regulate VEGF expression to facilitate angiogenesis; it may induce the expressions of NOS and HO, exerting vasodilation effect; it may activate the erythropoietin expression to induce erythrocytopoiesis and increase the oxygen carrying capacity[11]. In the present study, NSCs underwent the adenovirus mediated transfection of HIF-1α in vitro, and then these NSCs were transplanted into SCI rats.

Results demonstrated the HIF-1α protein expression in the injured spinal cord increased markedly after HIF-1α-NSC transplantation. The HIF-1α expression peak was postponed to 14 days after transplantation, and then HIF-1α expression reduced gradually, but was still at a high level ($P<0.01$). This suggests that transplantation of HIF-1α transfected NSCs may significantly up-regulate the HIF-1α expression in the injured spinal cord, which then increases the tolerance of spinal cord to ischemia and/or hypoxia and promotes angiogenesis, providing favorable environment for the repair of injured spinal cord.

NF is a neuron specific structure protein and can be classified into 3 categories on the basis of molecular weight: NF68, NF148 and NF200. Under normal condition, the cell body of neurons has no NF200 expression, and NF200 is mainly expressed in the axons. After SCI, axons became deformed and necrotic, and NF200 expression reduced significantly. During the repair of injured spinal cord, the NF expression increases significantly due to the presence of adjacent numerous factors. The increased NF then undergoes axonal transport into axons, and thus the NF positive axons increase, which is helpful for the regeneration of neurons[12]. Thus, detection of NF200 expression by immunohistochemistry can be used to reflect the severity of axonal injury and the degree of axonal repair. To preserve residual axons and promote the repair and regeneration of these axons are important goals in studies on SCI and for the treatment of SCI. Our results showed transplantation of NSCs could attenuate axonal injury and promote the regeneration and repair of injured axons. These effects were more obvious after transplantation of HIF-1α transfected NSCs, which is attributed to the high and stable expression of HIF-1α in the injured spinal cord. The treatment of SCI focuses on the microenvironment of axonal growth and the regeneration of neurons with injured axons[13]. HIF-1α can improve the microcirculation and then increases the tolerance of spinal cord to ischemia and/or hypoxia, which, together with NSCs, provides favorable microenvironment for the growth of axons and promotes the regeneration of injured axons. Our findings provide experimental basis for the treatment of SCI by transplantation of HIF-1α transfected NSCs.

GFAP is a major cytoskeletal protein in glial cells, and GFAP expression reflects the functional status of glial cells. Under normal condition, GFAP is dynamically regulated by astrocytes. After injury, the GFAP positive astrocytes proliferate, which then promotes the mitosis of astrocytes and the differentiation of primitive progenitor cells into mature astrocytes. CNS injury of any cause may induce the change in astrocytes. Experiments have shown that lysed astrocytes may release NGF, bNGF, IL-1, IL-3, IL-6 and TNF. After SCI, astrocytes may increase the number of intracellular organelles due to the requirement for neuronal growth factors. However, astrocytes have dual roles in the repair of injured spinal cord: at early stage, astrocytes became hypertrophic and proliferate to produce neurotrophic factors to promote neuronal repair; at late stage, hyperplasia of astrocytes may cause scar formation, which hinds the regeneration of neurons[14].

Our results showed HIF-1α trasnfected NSCs could significantly down-regulate GFAP expression at late stage of SCI, which then reduced the proliferation of glial cells and scar formation, providing beneficial environment for the regeneration of neurons and repair of injured spinal cord.

Taken together, transplantation of HIF-1α transfected NSCs may effectively induce high HIF-1α expression in the injured spinal cord, promote NF200 expression and reduce GFAP in the spinal cord at late stage, which then promote the regeneration of axons and reduce the proliferation of glial cells and scar formation, providing favorable microenvironment for the regeneration of neurons. However, the pathogenesis of SCI has involvement of multiple factors. In the present study, the pathogenesis of SCI was primarily explored, and there were some limitations in the present studies. Future studies are required to investigate the intrinsic mechanisms underlying the pathogenesis of SCI.

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